American Journal of Medical Genetics 66:77–80 (1996)

Validation Studies of *SNRPN* Methylation as a Diagnostic Test for Prader-Willi Syndrome

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Prader-Willi syndrome (PWS) is caused by absence of a paternal contribution of the chromosome region 15q11–q13, resulting from paternal deletions, maternal uniparental disomy, or rare imprinting mutations. Laboratory diagnosis is currently performed using fluorescence in situ hybridization (FISH), DNA polymorphism (microsatellite) analysis, or DNA methylation analysis at locus PW71 (D15S63). We examined another parent-of-origin-specific DNA methylation assay at exon α of the small nuclear ribonucleoprotein-associated polypeptide N gene (*SNRPN*) in patients referred with clinical suspicion of PWS or Angelman syndrome (AS). These included 30 PWS and 17 AS patients with known deletion or uniparental disomy status, and a larger cohort of patients (n = 512) suspected of PWS who had been analyzed previously for their methylation status at the PW71 locus. Results of *SNRPN* methylation were consistent with known deletion or uniparental disomy (UPD) status as determined by other molecular methods in all 47 cases of PWS and AS. In the larger cohort of possible PWS patients, *SNRPN* results were consistent with clinical diagnosis by examination and with PW71 methylation results in all cases. These data provide support for the use of *SNRPN* methylation as a diagnostic method. Because methylation analysis can detect all three major classes of genetic defects associated with PWS (deletion, UPD, or imprinting mutations), methylation analysis with either PW71 or *SNRPN* is an efficient primary screening test to rule out a diagnosis of PWS. Only patients with an abnormal methylation result require further diagnostic investigation by FISH or DNA polymorphism analysis to distinguish among the three classes for accurate genetic counseling and recurrence-risk assessment.

KEY WORDS: Prader-Willi syndrome, DNA methylation, *SNRPN*, genomic imprinting

INTRODUCTION

Prader-Willi syndrome (PWS) is now well-established as a paradigm for genomic imprinting in human disease [Nicholls, 1993]. DNA methylation is thought to play a role in controlling differential expression of the paternal and maternal alleles of imprinted genes [Li et al., 1993], and several parent-of-origin-specific methylation sites have been found in the PWS critical region of chromosome 15q11–q13, as demonstrated by Southern blot analysis using methylation-sensitive restriction enzymes. In normal individuals, both methylated maternal-specific and unmethylated paternal-specific alleles are observed, whereas only a maternal-specific allele is present in PWS patients [Driscoll et al., 1992; Dittrich et al., 1992, 1993; Glenn et al., 1993]. The diagnostic utility of differential methylation at one of these sites, PW71, has recently been demonstrated in a large patient series [Gillessen-Kaesbach et al., 1995], and has been confirmed in several other laboratories in smaller patient series [Young, 1995; van den Ouweland et al., 1995; Kokkonen et al., 1995].

Another differentially methylated site has been identified in the CpG island at the 5′ end of the small nuclear ribonucleoprotein-associated polypeptide N gene (*SNRPN*) [Sutcliffe et al., 1994]. At this site, the maternal chromosome appears to be completely methylated, while the paternal chromosome is completely unmethylated, allowing unambiguous identification of the

Received for publication January 9, 1996; no revisions.

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two parental alleles. However, the published data on *SNRPN* methylation have involved a very small number of PWS patients, and large-scale demonstration of its reliability as a clinical diagnostic test is needed.

We therefore investigated the *SNRPN* methylation pattern in a group of patients with known deletion or uniparental disomy (UPD) status, and in a large cohort of patients referred for possible PWS in whom PW71 methylation status was previously determined.

PATIENTS AND METHODS

Patients

SNRPN methylation analysis was performed on 559 patients submitted to our laboratories with clinical suspicion of PWS or AS. Of these, 47 had been analyzed previously by fluorescence in situ hybridization (FISH) and/or DNA microsatellite markers, and therefore their deletion or UPD status was known. In a larger cohort of 512 patients referred with clinical suspicion of PWS, 67 had been examined by a clinical geneticist experienced with PWS [Gillessen-Kaesbach et al., 1995], using the consensus diagnostic criteria for PWS [Holm et al., 1993]. All 512 patients in this cohort had been analyzed previously for their PW71 methylation status, allowing a direct comparison between the results of these two differentially methylated sites.

Methylation Analysis

DNA was extracted from peripheral blood collected in EDTA or ACD tubes or lymphoblast cells using standard methods. Early results indicated that blood samples collected in Na heparin tubes showed partial digestion patterns with *Not*I and therefore could not be used in the methylation assay.

Five μg of genomic DNA were digested with 50 u *Xba*I and 50 u *Not*I (New England Biolabs, Beverly, MA) in a volume of 500 μl. A control was used to assess *Not*I digestion as follows: 0.5 μg of DNA from a cosmid clone (c106, described in Sutcliffe et al. [1994]), which contains a *Not*I site but no *Xba*I site in the multiple cloning site of the vector (SuperCosI, Stratagene), were mixed with 30 μl of the patient digestion mixture. The patient sample and control were both incubated at 37°C overnight. The control digest was run on a small agarose gel and produced a distinct 7.6-kb fragment corresponding to the vector DNA when digestion was complete.

The digested patient DNA was concentrated down to 50 μl using Microcon 30 microconcentrators (Amicon, Inc., Beverly, MA), and separated on a 1.5% agarose gel for 2.5 hr. Southern transfer was performed onto Hybond N+ membrane (Amersham, Inc., Arlington Heights, IL) using standard overnight capillary transfer. To remove repetitive sequences, a 0.9-kb *Not*I fragment was cut from a 4.2-kb *Xba*I fragment containing the *SNRPN* exon α [Sutcliffe et al., 1994] (available from ATCC/NIH Repository, #95678, #95679). The *Not*I fragment was radiolabelled using the Rediprime DNA labelling system (Amersham, Inc.), and hybridized to Southern blots in a buffer containing 0.125 M Na_2HPO_4, pH 7.2, 0.25 M NaCl, 1 mM EDTA, 10% PEG-8000, and 7% SDS without preassociation with human placental DNA. Following hybridization, filters were washed to a final stringency of 0.1 × SSC/0.1% SDS at 65°C. Autoradiography was performed at −80°C with two intensifying screens for 6–20 hr (X-OMAT AR film, Eastman Kodak Company, New Haven, CT). The PW71 test was performed as described [Dittrich et al., 1993; Glenn et al., 1993].

RESULTS

Differential methylation at *SNRPN* was demonstrated using a double digestion with *Xba*I and the methylation-sensitive restriction enzyme *Not*I. The methylated (maternal) homolog was not digested with *Not*I and produced a 4.2-kb *Xba*I-*Xba*I fragment, while the unmethylated (paternal) homolog was digested at the *Not*I site and thus produced a smaller 0.9-kb *Not*I-*Not*I fragment (Fig. 1). Normal individuals therefore demonstrated both the 4.2- and 0.9-kb bands (normal methylation pattern), PWS patients demonstrated only the 4.2-kb maternal band with no paternal band present (PWS methylation pattern), and AS patients showed the opposite pattern, with the 0.9-kb paternal band present but the maternal band absent (AS methylation pattern). The probe gave a strong hybridization signal with overnight exposure, and very little hybridization background was present even without preassociation. Early experience showed occasional incomplete digestion with *Not*I, in which normal individuals had a weak paternal band (0.9 kb) and additional bands larger than the normal maternal band (4.2 kb). These partial digests were confined to DNA samples isolated from peripheral blood collected in sodium heparin tubes (for cytogenetic analysis), and were not observed in DNA isolated from EDTA blood collection tubes. Use of a control for *Not*I digestion as described in Patients and Methods provides an additional safeguard against this potential problem.

Results of *SNRPN* for the 47 patients whose deletion or UPD status had been previously determined by FISH and microsatellite analysis are illustrated in Figure 1 and summarized in Table I. Twelve PWS patients with known deletions or UPD showed the expected PWS methylation pattern of a single 4.2-kb maternal

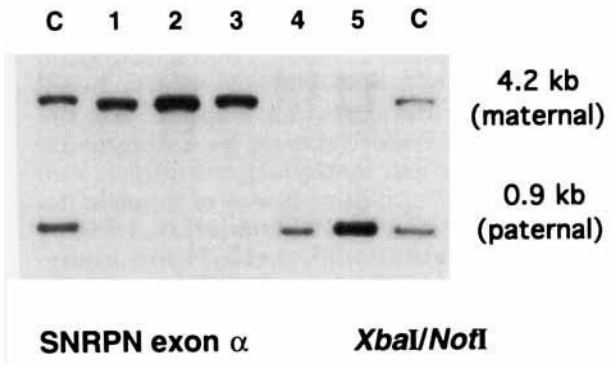

Fig. 1. Examples of *SNRPN* methylation assay on PWS and AS patients with known deletions or UPD. **Lane C**, normal control; **lane 1**, PWS patient with a paternal deletion; **lanes 2** and **3**, PWS patients with maternal UPD; **lanes 4** and **5**, AS patients with maternal deletions.

TABLE I. Summary of Patients With Known Deletions or UPD

Rule out PWS				Rule out AS	
Paternal deletion	Maternal UPD	IC[a]	Normal[b]	Maternal deletion	Normal[b]
4	8	3	15	5	12

[a] Patients with small paternal deletion at putative imprinting center (IC).
[b] No deletion or UPD detected by FISH/microsatellite analysis.

fragment. Three PWS patients known to have a very small paternal deletion at the putative imprinting center [Sutcliffe et al., 1994; Butler et al., 1996] also showed a PWS methylation pattern. Fifteen patients originally referred from outside sources with clinical suspicion of PWS in whom no deletion or UPD was found showed normal methylation patterns. Five AS patients with known maternal deletions showed only the 0.9-kb paternal fragment for *SNRPN*. Twelve other patients referred for possible AS but who did not have a deletion or UPD present showed a normal methylation pattern. Thus, there was no discrepancy between prior molecular analyses and results of the *SNRPN* methylation analysis.

To further establish the validity of *SNRPN* methylation as a diagnostic assay, we tested a total of 512 patients with clinical suspicion of PWS who had been previously analyzed for methylation status at PW71. Representative results are shown in Figure 2, and complete results are summarized in Table II. Of the 512 patients previously studied, 495 had sufficient high-quality genomic DNA for further analysis. For *SNRPN*, 161 patients showed a PWS methylation pattern, while 334 showed normal methylation. These results are 100% concordant with previous results for PW71.

In this large cohort, 67 patients were examined clinically, and 36 were determined to have PWS. Of these, all showed a PWS methylation pattern, while the other 31 patients showed a normal pattern. These results indicate complete concordance between clinical diagnosis of PWS by an experienced geneticist and *SNRPN* methylation pattern.

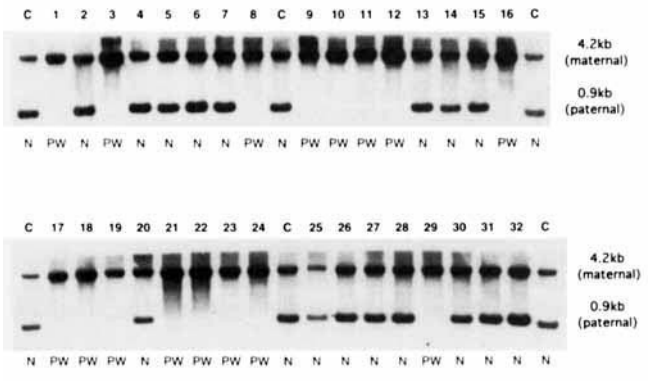

Fig. 2. Examples of *SNRPN* methylation studies on the large cohort of patients referred with clinical suspicion of Prader-Willi syndrome. **Lane C**, normal control; **lanes 1–32**, representative samples of patients. N, normal pattern with both maternal and paternal bands; PW, PWS pattern with maternal band only.

DISCUSSION

The clinical diagnosis of PWS can be difficult due to the presence of many subtle or nonspecific manifestations, including neonatal hypotonia, failure to thrive, global developmental delay, and mental retardation. Consensus clinical diagnostic criteria were established recently for PWS to aid in this diagnosis [Holm et al., 1993]. For laboratory diagnosis, FISH has become the method of choice for identification of interstitial deletions of chromosome 15q11–q13 [Kuwano et al., 1992], whereas DNA analysis with microsatellite markers is used for detection of UPD [Mutirangura et al., 1993]. However, each of these methods has limitations, e.g., FISH cannot detect UPD, and microsatellite analysis requires parental blood samples. Neither FISH nor DNA polymorphism analysis will detect rare patients with imprinting mutations who may have small deletions or point mutations in the imprinting center region. As an initial screening test, methylation analysis has the advantage of detecting all three of these major classes of molecular defects involved in PWS (deletions, uniparental disomy, and imprinting mutations) without the need for parental blood.

In the present study, we demonstrated concordant results between *SNRPN* methylation and deletion or UPD status determined by other molecular methods in a group of 47 patients referred with clinical suspicion of PWS or AS. In a larger group of 512 patients referred to rule out PWS, the *SNRPN* methylation pattern was completely concordant with methylation results at locus PW71, the only methylation site previously validated as a diagnostic test for PWS [Gillessen-Kaesbach et al., 1995]. In 67 patients examined clinically, the methylation results for both PW71 and *SNRPN* were consistent with the clinical diagnosis, using the recently developed consensus criteria for PWS [Holm et al., 1993]. Therefore, *SNRPN* methylation analysis, similar to PW71, is a reliable diagnostic test for PWS.

TABLE II. Comparison of Methylation Analysis between PW71 and *SNRPN* in Patients Referred to Rule Out PWS

	PW71	*SNRPN*
Total samples	512	512
Excluded	0	17[a]
Total analyzed	512	495
PWS pattern	167	161[b]
Normal pattern	345	334[b]

[a] Insufficient genomic DNA available.
[b] Complete concordance with PW71 in all cases.

In our series of 47 patients with known deletion or UPD status based on other molecular methods, concordant results were obtained for *SNRPN* methylation using genomic DNA from either peripheral blood or isolated from lymphoblastoid cell lines (n = 18). Some previous reports [e.g., van den Ouweland et al., 1995] suggested that PW71 may show occasional discrepancies using DNA from cultured tissues. Additional data comparing the methylation patterns for PW71 and *SNRPN* in various cell and tissue types (e.g., lymphoblastoid cells, skin fibroblasts, amniotic fluid cells, and chorion villus samples) are needed to determine their diagnostic utility for each of these tissues.

In an individual patient with a relatively certain clinical diagnosis of PWS, FISH analysis may be the most efficient diagnostic test, as approximately 70% of patients will have a deletion detectable by FISH. However, since a very high percentage of patients are referred for laboratory diagnosis to rule out PWS based on only a few relatively common abnormalities, DNA methylation analysis is the most efficient single test for ruling out a PWS diagnosis. As reported previously, methylation studies may also prove useful for screening hypotonic infants for possible PWS, thus avoiding muscle biopsy and other expensive diagnostic assays [Gillessen-Kaesbach et al., 1995].

A few patients with signs of PWS have been described with balanced translocations within or distal to *SNRPN*, who had normal methylation patterns [Conroy et al., 1995; Sun et al., 1994; Schwartz et al., 1995]. These cases emphasize the importance of conventional cytogenetic analysis in parallel with DNA methylation analysis, to detect these rare translocation cases and also to detect other chromosome abnormalities in patients who do not prove to have PWS. Although methylation is strongly recommended as an initial diagnostic test to rule out PWS, it is important to follow up abnormal methylation results by FISH or microsatellite analysis to determine the precise etiology (deletion, UPD, or imprinting mutation) for complete genetic counseling and recurrence-risk assessment.

ACKNOWLEDGMENTS

The authors thank Sabine Kaya-Westerloh for technical assistance. This study was supported in part by grants from the Deutsche Forschungsgemeinshaft (to B.H.) and from the Beneficia Foundation (to D.H.L).

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